Bright, highly water soluble triazacyclononane europium complexes
to detect ligand binding with time resolved-FRET microscopy**

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Abstract: Luminescent europium complexes are used in a broad range of applications due to their particular emissive properties. Here we describe the synthesis and application of bright, highly water soluble and negatively charged sulphonic- or carboxylic acid derivatives of p-substituted aryl-alkynyl triazacyclononane complexes. Introduction of the charged solubilizing moieties suppresses cellular uptake or adsorption to living cells making them applicable for labeling and performing assays on membrane receptors. These europium complexes are applied to monitor fluorescent ligand binding on cell surface proteins with time-resolved Förster Resonance Transfer assays in plate based format and using TR-FRET microscopy.

Luminescent lanthanide complexes are used in an impressive number of applications, such as sensors,[1, 2] bioassays,[3], or OLEDs,[4], due to their peculiar photophysical properties. Their use in luminescence based cellular imaging purposes has been particularly interesting, since background fluorescence from cells can be completely rejected using time-gating [1, 2, 5, 6]. A key challenge remains to develop bright probes to address specific biological processes in living cells or for cell tissue samples.[3, 7, 8] Recently, we have developed very bright europium triazacyclononane (TACN) complexes that stain various intracellular compartments, such as mitochondria, following cell uptake by macropinocytosis.[9-12] Such behavior is disadvantageous, if you seek to develop assays for membrane proteins, such as G-protein coupled receptors [13, 14]. With all the previously developed complexes,[9-12] the non-specific interactions[5, 13] due to the probe adsorbing to the cell membrane or proteins within, cellular uptake or the labeling of immature proteins within the cytosol reached values not compatible with homogeneous time resolved fluorescence (HTRF) bioassays.[10] Here we describe the synthesis of europium TACN complexes and their bio-conjugates with improved water solubility that are incapable of entering or adhering to cells at concentrations <1 μM. The bioconjugates were evaluated in TR-FRET ligand binding assays on living cells expressing the cholecystokinin2 (CCK2) receptor both in plate based format as well using TR-FRET microscopy.

Since the cell membrane consists of phospholipids and is considered to be negatively charged, we developed TACN complexes, which are highly water soluble and negatively charged at physiological pH (Scheme 1). We reasoned that this should reduce interaction with the cell membrane due to repulsive Coulombic interactions. Moreover, the hydrophilic nature of the sulphonate and carboxylate groups should mask the inherent hydrophobic nature of the three aryl-alkynyl groups. The emission spectrum and photo-physical properties of the parent complexes e.g. [EuL3] and the ring-substituted analogues such as [EuL2] do not change significantly, indicating that the local C3 symmetry around the Eu3+ ion is not compromised. Hence, bio-conjugation of the complexes was initiated by using derivatives substituted at the ring carbon of the TACN macrocycle, e.g. those based on [Eu.L3]. This approach avoids varying the ring N-substituents, as asymmetry in the aryl-alkynyl antennae complicates the synthesis.[9]

[†] V. Sadovnikova was supported by the TOPBIO program (FP7 ITN Marie Curie 264362)

[**] We thank the ERC (FCC-266804) and the Fonds Unique Interministériel for support

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201xxxxxx

Scheme 1. Structures of parent and C-substituted Eu complexes

The synthesis of these complexes was undertaken in a modular manner, using three key intermediates (Scheme 2). The C-
substituted TACN intermediate, 1, was prepared using an adaptation of the literature method (see SI), wherein copper binding to the three ring N atoms allows the remote primary amine to react selectively with BocO. Copper is rapidly removed by bubbling H2S through the solution, and the three ring N atoms were alkylated with the known pyridyl mesylate, 2 (Scheme 2) [10,11]. The benzylguanine moiety was introduced following reaction of the NHS ester, 3, with the primary amine on the ring substituent that had been unmasked by de-protection of the BOC precursor (TFA, RT).

Scheme 2. Retrosynthesis of the target Eu conjugates

The water solubility of these and related complexes was compared by assessing the partitioning coefficient (logP) of the complexes in water/octanol mixtures. Three equimolar solutions of complex were prepared in MeOH. The solvent was removed under reduced pressure and the resulting solid was dissolved and stirred for 24 h in 0.9 mL of a mixture of water/octanol (2:1, 1:1, 1:2) giving a total concentration of approximately 2 μM. After equilibration, an emission spectrum for each layer was recorded in MeOH (50 μL of solution in 1 mL of MeOH). For each mixture, the logP value was calculated (see SI).

Table 1: Photophysical properties and logP values of the [Eu.L] complexes (295K, H2O)

<table>
<thead>
<tr>
<th>Complex</th>
<th>λmax / nm</th>
<th>τ / ms</th>
<th>Φ / %</th>
<th>ε / mM⁻¹cm⁻¹</th>
<th>logP</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Eu.L5]</td>
<td>330</td>
<td>1.03</td>
<td>24</td>
<td>58</td>
<td>+1.4</td>
</tr>
<tr>
<td>[Eu.L7]</td>
<td>330</td>
<td>1.01</td>
<td>26</td>
<td>58</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

The introduction of sulphonate or carboxylate groups enhances water solubility significantly, and reduces the logP value from +1.4 to -2.2. The absorption and emission spectrum (Figure 1) and the excited state lifetimes hardly change when compared to the parent compounds [13], although the overall luminescence quantum yield in water drops slightly suggesting that the energy transfer from the ligand ICT excited state to Eu³⁺ is not quite as efficient in water as in methanol. [19]

After solving the problem of water solubility, we evaluated the complexes using SNAP-tag technology. [17-21] on the cholecystokinin-2 (CCK2) receptor, a G-protein coupled receptor, which has elevated expression levels when transiently transfected in HEK293 cells. The labeling of the benzylguanine-derivatives [Eu.L] [Eu.L₄], and [Eu.L] on living HEK293 cells or HEK293 cells expressing the SNAP-tagged CCK2 (SNAP-CCK2) [21] was measured by monitoring the time gated luminescence intensity at 620 nm (Figure 2b-d).

Significant labeling of non-transfected HEK293 cells occurs with the less hydrophilic compound [Eu.L₅], whereas with [Eu.L₄] and [Eu.L] this non-specific labeling is negligible. These excellent results tempted us to perform a TR-FRET ligand binding assay using a fluorescent agonist of the CCK2 receptor.

Addition of increasing concentrations of the red fluorescent-agonist of CCK2, red-CCK(26-33) [21] to [Eu.L] labeled living SNAP-CCK2 cells and measuring the time resolved FRET at 665 nm results in a saturating binding curve (Figure 2e). Note that adding 10 μM of the natural agonist CCK-(26-33) at the different concentrations of fluorescent ligand and the TR-FRET signal is annihilated due to the competition of non-labeled compound. By applying a one-site binding model to the specific binding data, a dissociation constant (Kd) of the red agonist of 8 nM was calculated, similar to data reported in the literature. [21] A TR-FRET competition binding assay shows that binding of 10 nM of red-CCK(26-33) is completely reversed in a dose dependent way by adding saturating amounts of the antagonist PD135158. The inhibition constant (KI) is calculated to be 6 nM, in accordance with literature values. [21]

Figure 1 Absorption and total emission spectra for [Eu.L] (295K, water)

Figure 2. a) Cartoon showing a TR-FRET ligand binding assay on HEK293 cells expressing SNAP-CCK2 (adapted from ref 3). b,c,d Saturation curves for labeling HEK293 expressing SNAP-CCK2 and non-transfected cells at the cell surface using TR-detection (620 ± 5 nm, 60-460 μs with [Eu.L], [Eu.L₄] and Eu²⁺ respectively. e) Binding of red-CCK(26-33) to HEK293 cells expressing SNAP-CCK2 labeled with 200 nM of [Eu.L] monitored by following the 665/620 x10000 ratio. Non specific signal is measured by adding 10 μM of unlabeled CCK(26-33). f) TR-FRET competition binding assay, monitoring the dose-
Since the non-specific labeling was suppressed we could also monitor the labeling of SNAP-CCK2 with Eu.L\(^7\) using time resolved microscopy. HEK293 cells (50k) were plated on a lab-tek slide, transfected with SNAP-CCK2 plasmid and incubated for 2 days after which they were labeled with 200 nM of Eu.L\(^7\) during 1h. Using a delay of 100 µs and a gate-time of 2 ms, a typical TR-image recorded with a filter of 615±10 nm is shown in Figure 3a. Labeling clearly occurs at the cell surface and not within the cell. Only recently has lanthanide based time resolved FRET microscopy [3, 9, 22, 23] been reported, mainly using a Tb-complex. [3, 24, 25] To pursue the venture with these complexes, we tested whether this technology can also be applied with these europium complexes to monitor ligand-receptor interactions on GPCR. Since a band-pass filter at 615 nm was used to detect the TR signal, we could also use the same set-up to monitor the time-resolved FRET signal at 670 nm. Similar to the plate-based assay (Figure 2e), the red fluorescent agonist of CCK2 was added to the cells labeled with Eu.L\(^7\). The TR-FRET image, recorded at 670±20 nm suggests the formation of vesicles, indicating that many of the receptors previously expressed at the cell surface are now internalized together with the red-fluorescent agonist through a ligand induced internalization process. The image data for both the europium and the TR-FRET channel for the conditions with or without CCK(26-33)-red (see Figure 3) reveals that the TR-FRET signal under the same imaging conditions is well above the intensity due to donor bleed-through in the TR-FRET channel. Similarly the Eu-channel intensity is decreased due to the FRET process.

![Figure 3](image)

In conclusion, we have developed bright, kinetically stable, highly water soluble Eu\(^{3+}\) complexes whose emissive properties are unchanged following bio-conjugation. The C-substitution of the TACN ring with an aminooalkyl group (derived from S-lysine), creates a useful synthon that is easily functionalized with different antennae ligands\(^{[26]}\) for use in a range of different applications. By introducing sulfonate or carboxylate groups onto the aroyl-alkynyl antennae, non-specific labeling of living HEK cells is suppressed, permitting the detection of the GPCR CCK2 using derivative Eu.L\(^7\). Using time-resolved detection, the TR-FRET ligand binding assays both on a plate reader and with TR-FRET microscopy are possible. The low non-specific interactions of these complexes with cells will be a great advantage for the development of novel HTRF assays where specific biological interactions are studied. Furthermore, by introducing different electron donating moieties on the aryl-ring, we are developing biocjugates whose electronic absorption bands are bathochromically shifted\(^{[26]}\) thereby improving their brightness for use in time-resolved confocal or two-photon microscopy.

**Experimental Section**

**MATERIAL AND METHODS**

**Reagents**

The Tag-lite labeling medium (ref. LABMED), the SNAP-CCK2 plasmid for transient transfection of CCK2 receptors (ref. PSNAPCCK2) and (CCK(26-33)-red) (ref. LO013RED) were obtained from Cisbio Bioassays. The CCK2 receptor antagonist PD135158 was purchased from Tocris. CCK(26-33) was obtained from Almac (Craigavon, UK). The 96-well plates were purchased from Greiner Bio-One (ref. 655086, Monroe, NC).

**Cell culture**

HEK293 wild-type cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) glutamax (1996-021: Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 2mM HEPES.

**Transfection procedure**

Transfection was performed in 96-well plates using 100,000 cells per well according to a previously described protocol\(^{[21]}\). Prior to cell seeding, wells of the plates were precoated with 50 µL of poly-L-ornithine for 30 min at 37 °C. The transfection mixture (per one well) was prepared by adding 100 ng of the SNAP-tag-CCK2 plasmid to 49 µL OptiMEM medium and 0.8 µL of Lipofectamine 2000 (Invitrogen) and incubated for 20 min at room temperature prior addition in plates. Subsequently, 100 µL of HEK 293 cells at a density of 10⁵ cell/mL was distributed in each well of the plates. Plates were incubated overnight at 37 °C under 5 % CO2.

**Receptor labeling**

For the labeling of SNAP-CCK2 expressed HEK293 cells with Eu.L\(^{5-7}\) a previously described protocol\(^{[21]}\) was used. A concentration series ranging from 0-500 nM was prepared in Tag-lite labeling medium. After incubation the transfection mixture was removed from 96-well plates, and cells were treated with 50 µL of the prepared solutions and incubated for 1h at 37 °C under 5% CO2. The unreacted compounds were removed by washing each well 4 times with 100 µL of Tag-lite labeling medium.

**Fluorescent ligand binding assay**

Affinity of red-CCK(26-33) for the CCK2 receptors was determined by incubating labeled cells with increasing concentrations of the fluorescent ligand. The non-specific signal for each ligand concentration was determined by adding an excess of the corresponding unlabeled compound (10 µM CCK(26-33)). In plates containing labeled cells with 100 µL of Tag-lite labeling medium 20 µL of unlabeled compound (CCK(26-33)) was added followed by addition of 20 µL of fluorescent ligand red(CCK(26-33)). Plates were incubated at room temperature for 2 hours before the signal detection.

**Competition binding assay**

In competitive binding experiments a fixed concentration of fluorescent ligand (10 nM red-CCK(26-33)) was used in presence of increasing concentrations of antagonist PD135158. In plates containing labeled cells with 100 µL of Tag-lite labeling medium was added 20µL of PD135158 followed by the addition of 10
μL of fluorescent ligand (red-CCK(26-33)). Plates were incubated at room temperature for 4 hours before the signal detection.

**Signal detection and data analysis**

Signal detection was performed on PHERAstar FS plate reader (BMG LABTECH, Champigny-sur-Marne, France) at 620 nm and 665 nm (in TR mode : delay, 60 µs ; time gate, 400 µs) upon 337 nm laser excitation. Recorded data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Specific binding was determined by subtracting nonspecific signal from the total signal. Kᵣ values of the fluorescent ligand were obtained from the saturation curve of the specific binding. Kᵣ values were calculated from competition assay experiments according to the Cheng and Prusoff equation.¹²

**TR-FRET Microscopy**

Lab-Tek 8 chamber slide system was precoated with 200 µL polyl-ornithine and incubated for 30 min at 37 °C. The slide was washed with 200 µL PBS, HEK 293 cells were plated at a density of 5 × 10⁵ c/w and incubated overnight at 37 °C under 5% CO₂. The following day transfection was performed with 100 µl optiMEM medium, 0.8 µL of Lipofectamine 2000 and 1.2 µg SNAP-CCK2 plasmid (per well). The Lab-Tek slide was further incubated for 2 days at 37 °C under 5% CO₂. After removal of the medium SNAP-CCK2 receptors were labeled with 200 nM of [Eu.L]³⁺ for 1h at 37 °C and washed 2 times with tag-lite medium. For the FRET signal detection 10 nM of red-CCK(26-33) was added. 2µg/mL of Hoechst 33342 (per well) was added and after 20 min incubation at room temperature and washing twice with Tag-lite medium the Lab-Tek slide was observed with a Zeiss oil immersion objective (40x, 1.3 F-Fluar) on the Zeiss Axiovert 200M TR-FRET inverted microscope equipped with a pulsed nitrogen laser (337 nm, 30 Hz) and a cooled intensified CCD camera Pi Max 1024X1024 Genill. For the luminescence imaging of [Eu.L]¹⁺ 615 ± 10 nm band-pass filter was used with a 5nm of 100 µm a 5nm of 200 µm and at 60 gates per exposure. TR-FRET images were collected with a 670 ± 20 nm band-pass filter using the same set up as written above. The recorded data were analyzed using ImageJ software.

Received: (will be filled in by the editorial staff)  
Published online on (will be filled in by the editorial staff)

**Keywords:** Europium • TR-FRET • GPCR • Imaging • macrocycle

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Bright, highly water soluble triazacyclononane europium complexes to detect ligand binding with time resolved-FRET microscopy

Bright and selective: hydrophilic Eu(III) complexes have been created that suppress non-specific binding and permit their use in bio-conjugates using time-resolved assays exemplified for G-protein coupled receptor antagonists.